

1 **First isolation of a new species of *Leishmania* responsible for**
2 **human cutaneous leishmaniasis in Ghana and classification in**
3 **the *Leishmania enriettii* complex**

4
5 **Godwin Kwakye-Nuako^{a,b}, Mba-Tihssommah Mosore^{c,d}, Christopher Duplessis^{c,1},**
6 **Michelle D. Bates^a, Naiki Puplampu^c, Israel Mensah-Attipoe^e, Kwame Desewu^f,**
7 **Godwin Afegbe^g, Richard H. Asmah^h, Manal B. Jamjoom^{a,2}, Patrick F. Ayeh-Kumi^{e,h},**
8 **Daniel A. Boakye^d, Paul A. Bates^{a*}**

9
10 ^aDivision of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster
11 University, Lancaster, UK

12 ^bDepartment of Biomedical and Forensic Sciences, School of Biological Sciences,
13 University of Cape Coast, Cape Coast, Ghana

14 ^cU.S. Naval Medical Research Unit No. 3 (NAMRU-3), Noguchi Memorial Institute for
15 Medical Research, University of Ghana, Legon, Accra, Ghana

16 ^dDepartment of Parasitology, Noguchi Memorial Institute for Medical Research, University
17 of Ghana, Legon, Accra, Ghana

18 ^eDepartment of Microbiology, University of Ghana Medical School

19 ^fGhana Health Service, Accra, Ghana

20 ^gDisease Control Unit, Ministry of Health, Ho

21 ^hSchool of Allied Health Sciences, College of Health Sciences, University of Ghana, Korle-
22 Bu, Accra

23
24 ¹Current address: Naval Medical Research Center, 503 Robert Grant Avenue, Silver
25 Spring, MD 20910, USA

26 ²Current address: Department of Medical Parasitology, Faculty of Medicine, King Abdul
27 Aziz University, Jeddah, Saudi Arabia

28

29 *Corresponding Author: Prof. Paul Bates, Division of Biomedical and Life Sciences, School
30 of Health and Medicine, Lancaster University, Lancaster, LA1 4YQ UK. Tel.: +44 1524
31 593718; fax: +44 1524 592658; e-mail address: p.bates@lancaster.ac.uk

32

33 **Disclaimer:** The opinions stated are those of the authors and do not represent the official
34 position of the US Department of Defense, local country Ministries of Health, Agriculture,
35 or Defense, or other contributing network partner organizations. Mention of any
36 commercial product does not imply DoD endorsement or recommendation for or against
37 the use of any such product. No infringement on the rights of the holders of the registered
38 trademarks is intended.

39

40 **Abstract**

41 An active case detection approach with PCR diagnosis was used in the Ho District of the
42 Volta Region that identified individuals with active cutaneous leishmaniasis. Three isolates
43 were successfully cultured and DNA sequences from these were analysed (ribosomal
44 RNA ITS1; RPL23a intergenic; RNA polymerase II large subunit), showing them to be
45 *Leishmania*, identical to each other but different to all other known *Leishmania* species.
46 Phylogenetic analysis showed the parasites to be new members of the *Leishmania enriettii*
47 complex, which is emerging as a possible new subgenus of *Leishmania* parasites
48 containing human pathogens.

49

50 **Keywords:** Ghana, *Leishmania*, cutaneous leishmaniasis, phylogeny

51

52

53 Cutaneous leishmaniasis (CL) is a significant emerging disease in the Volta Region
54 of Ghana (Kweku et al., 2011) and has become sufficiently common to acquire a local
55 name, “agbamekanu”, with estimates of high prevalence in some communities. Reported
56 infections have occurred mainly in the Ho District, a moist semi-deciduous forest zone with
57 villages dotted around the district capital and an estimated population of 235,000 (Ghana
58 Statistical Service, 2002 Population and Housing Census). From clinical signs, 8,533
59 cases were recorded in Ho District in 2002 and 2003, which represents ~3-4% of the
60 population, and in surveys of schoolchildren prevalence of leishmanial-like lesions ranged
61 from 12.2 to 32.3 % (Kweku et al., 2011). However, the number of cases with
62 parasitologically confirmed diagnoses is very small (Fryauff et al., 2006; Villinski et al.,
63 2007), and prior to the current study parasites causing CL in Ghana had never been
64 isolated into culture. The identity of the species responsible remain uncertain, one PCR-
65 confirmed case from a biopsy was identified as *L. major* by ribosomal RNA ITS1
66 sequencing (Fryauff et al., 2006), but in a second study conducted in the same area
67 further biopsies found no match to any known *Leishmania* species (Villinski et al., 2007).
68 Regarding transmission, leishmaniasis is a vector-borne disease usually transmitted by
69 sand flies but the majority caught to date in Ghana have been various *Sergentomyia*
70 species, which are not generally regarded as likely vectors of human leishmaniasis
71 (Ready, 2013). However, low numbers of the possible vectors *Phlebotomus rodhaini* and
72 *P. duboscqi* were found (Fryauff et al., 2006). It has been recently reported that *L. tropica*
73 DNA was found in *Sergentomyia hamoni* and *S. ingrami*, and *L. major* DNA in *S. ingrami*,
74 in flies collected from the endemic region (Nzelu et al., 2014). However, the finding of
75 parasite DNA by itself does not prove vector status, as blood meal infections can persist in
76 non-vectors for some days but will not result in established transmissible infections
77 (Ready, 2013). The current study was undertaken to isolate and characterize parasites
78 causing CL in Ghana. Cultures were established for the first time, and here we present

evidence that these represent a new species of *Leishmania*, which is related to several other species grouped within the *Leishmania enriettii* complex. These parasites are the first new human-infective *Leishmania* species to be isolated in Africa for over 40 years.

Fifteen villages in Ho District with previous records of suspected or reported cases of CL were initially visited, and of these five with recent cases were followed up: Matse-Lotus, Sokode-Gbogame, Dodome-Doglome, Dodome-Awiaususu, and Lume-Atsyame. The study was assessed by and approved by the University of Ghana Medical School Ethical and Protocol Review Board, Protocol Identification Number MS-Et/M.6.1 – P.3/2006-07 and the Noguchi Memorial Institute for Medical Research Institutional Review Board, CPN 062/11-12. The purpose of the study and the procedures to be followed were explained and written informed consent was obtained from all participants or their legal guardians prior to any intervention. A total of 68 people with suspected CL were seen, of these 44 were recruited into the study and 41 subsequently confirmed as infected with *Leishmania*, 38 by PCR from dermal scrapings and 3 cases by successful culture and DNA sequence analysis (further details of sampling and other methods in Supplementary Data S1).

Typical households included 10 to 14 inhabitants, and usually 1-2 people per household had healed lesions on the body suggestive of past CL. Two sets of PCR primers were used for diagnosis: Mincr2 and Mincr3 are derived from the conserved region of *Leishmania* species minicircle DNA of the parasite kinetoplast, generating a product of 120 bp (Degraeve et al., 1994; da Silva et al., 2004); R221 and R332 are *Leishmania*-specific and amplify a region of the 18S rRNA gene, generating a product of 603 bp (van Eys et al., 1992; Meredith et al., 1993). The Mincr2/Mincr3 primer pair demonstrated better efficacy, amplifying 38 out of the 41 dermal scraping samples taken (93 %; Fig. 1A, Supplementary Fig. S1A), with the R221/R332 pair amplifying 27 out of the 41 samples (66 %; Fig. 1B, Supplementary Fig. S1B). All samples that were positive with R221/R332 were also positive with Mincr2/Mincr3, no additional positive cases were discovered with R221/R332.

105 Thus the R221/R332 primers detected *Leishmania* in fewer samples, which is consistent
106 with the lower copy number of their targets at about 160 copies per genome (van Eys et
107 al., 1992), compared to ~10,000 copy number of the minicr1/minicr2 targets (Degraeve et
108 al., 1994). The 3 negative samples from dermal scrapings are either true negative non-
109 leishmanial skin lesions that can arise from a number of other causes, or are *Leishmania*
110 infections below the level of detection. There was no evidence of other differential
111 diagnoses from Buruli ulcer, yaws or cutaneous fungal infections amongst the participants.
112 Use of dermal scrapings for diagnosis of *Leishmania* is a minimally invasive sampling
113 method for CL, and another useful aspect of this study was the ability of the primers to
114 detect *Leishmania* from the lesion material on FTA cards, without the need for separate
115 isolation of DNA from clinical samples.

116 Three lesion aspirate samples from separate individuals were used to establish
117 promastigote cultures. Sloppy Evans semi-solid medium was prepared by mixing 350 ml of
118 Locke's solution (9 g NaCl, 0.42 g KCl, 0.4 g CaCl, 0.2 g NaHCO₃, 1 g Glucose and dH₂O
119 to 1 litre) with 1.3 g Agar No 1, 2 g bacteriological peptone, and 0.2 g Beef extract (Bovril),
120 which was autoclaved, after which 50 ml defibrinated sterile rabbit blood was added, mixed
121 and 2 ml aliquots dispensed into sterile bijou tubes. Lesion aspirates were transferred into
122 2ml volumes of Sloppy Evans, incubated at 26 °C and checked every 48 hours for up to a
123 month by phase contrast microscopy. Upon observing promastigotes, aliquots were
124 transferred into liquid culture medium comprised of Medium 199 (Life Technologies 12350-
125 039) supplemented with 20 % (v/v) fetal bovine serum, BME vitamins (Sigma B6891) and
126 25 µg/ml gentamicin sulphate. Cultures were expanded and sub-passaged as required and
127 promastigotes cryopreserved in 7.5 % glycerol at -80 °C and liquid nitrogen. The WHO
128 codes for these isolates are MHOM/GH/2012/GH5;LV757, MHOM/GH/2012/GH10;LV758
129 and MHOM/GH/2012/GH11;LV759, hereafter referred to as GH5, GH10 and GH11,
130 respectively. Amplification of the ITS1 sequence from each isolate was performed, and the

131 resulting sequences were found to be very similar or identical to each other and to the
132 sequence previously reported by Villinski et al. (2007) derived from CL lesion biopsies,
133 showing 99.2-99.6% identity (Supplementary Fig. S2). These sequences are more similar
134 to each other than to any other known *Leishmania* sequence, and the degree of similarity
135 is what would be expected for ITS1 sequences from different isolates of the same species.

136 Infection in confirmed cases was predominantly in younger age groups (~58% were
137 up to 10 years old), although the age range included from infants to adults above fifty
138 years (Supplementary Table S2). The majority of these were recent infections with open
139 sores in which the observed lesions were either crusted or ulcerated (Fig. 2). No nodular
140 or papular forms were observed. The lesions of the majority of participants were circular,
141 except for one person who had both circular and oval lesions. Their appearance was
142 relatively uniform, perhaps indicating the presence of a single species responsible for CL. On
143 the other hand, there is often wide variation in the appearance of lesions even with a
144 single species (Reithinger et al., 2007). More work is required to see if any pattern or
145 significant variation of lesion form emerges in Ghana. The number of lesions per person
146 ranged from 1 to 3, and no multiple diffused lesions were observed on any of the
147 participants. In all, a total of 51 lesions were found on confirmed cases, of these 33
148 individuals had single lesions, 6 had two lesions and 2 had three lesions. The average size
149 in diameter and reported age of the lesions (according to the participants) were 11.1 mm
150 and 3.9 weeks, respectively. The sites of the lesions were classified into five regions and
151 approximately half (53%), were located on the head (Supplementary Table S3). This could
152 simply mean that the vector preferred uncovered parts of the body, consistent with the fact
153 that the vector if active at night will bite the exposed head parts, since people will typically
154 cover the body but not the head when asleep, or be due to some behavioral property of
155 the vector. There were no reports of severe clinical symptoms accompanying the lesions,
156 there were a few reports of low grade fever, and slight pain and discomfort associated with

157 the ulcers. The recruitment of participants was not evenly distributed through time, with the
158 number of cases detected increasing from July, peaking in September and declining to
159 November (Supplementary Fig. S3). This peak in recruitment is approximately three
160 months after the peak in rainfall in June, the rainy season running from May to November,
161 which could have contributed to increased transmission by elevating vector numbers. This
162 is similar to findings in Afghanistan, where a rise in numbers of CL in various age groups
163 from August to November and then a decline in December were reported (Faulde et al.,
164 2007). This pattern is also consistent with the typical evolution of CL, where a lesion will
165 develop over a period of 2-3 months before beginning to heal and form scar tissue. There
166 were old scars in some individuals, which were dappled, somewhat depressed and de-
167 pigmented, suggestive of earlier CL in the communities (Okwori et al., 2001; Mendonca et
168 al., 2004).

169 DNA was isolated from the three isolates established in culture. Initial identification
170 of these was performed using a PCR-RFLP test we have developed that amplifies across
171 a single copy intergenic region of approximately 1.5 kb between the RPLS7A and RPS7B
172 genes (*L. major* chromosome 1) using primers AM1/AM2. This target has been found to
173 give species-specific banding patterns in all of a wide range of *Leishmania* species
174 examined to date. A single product was generated from each of the three isolates, GH5,
175 GH10 and GH11, which when digested with *MspI* generated identical bands in each case
176 that were completely different to those found with reference strains (Fig. 1C). In addition to
177 the data shown, the banding pattern of the Ghanaian isolates was different to that seen in
178 a wide range of additional reference strains examined, including *L. infantum*, *L. donovani*,
179 *L. amazonensis*, *L. mexicana*, *L. braziliensis* and *L. guyanensis*. These results are
180 consistent with the Ghanaian parasites representing a new species. To investigate this
181 further, PCR was performed on GH5, GH10 and GH11 DNA using the BN1/BN2 primers
182 that amplify a single copy intergenic region of 450-500bp between two RPL23a genes (*L.*

183 *major* chromosome 6), followed by sequencing, as used previously for phylogenetic
184 analysis (Dougall et al., 2011). Each isolate generated a 468bp product of identical
185 sequence. Analysis of these sequences and comparison with the homologous sequences
186 from a range of other *Leishmania* species in a maximum likelihood (ML) tree is shown in
187 Figure 3A. The three established subgenera, *Leishmania* (*Leishmania*), *Leishmania*
188 (*Sauroleishmania*) and *Leishmania* (*Viannia*) were all supported with high bootstrap
189 values, as was the *L. enriettii* complex and the *paraleishmania*. The Ghanaian parasites
190 clustered within the *L. enriettii* complex, which includes *L. enriettii* itself (Lainson, 1997), *L.*
191 *martiniquensis* (Desbois et al., 2014), a new as yet un-named species from Australia (AM-
192 2004) (Rose et al., 2004) and "*L. siamensis*" (Bualert et al., 2012). It should be noted that
193 "*L. siamensis*" has not been formally described, despite appearing in the literature several
194 times, and is therefore currently a *nomen nudum*, hence the name is used in quotation
195 marks. Further, although the majority of "*L. siamensis*" appear to be *L. martiniquensis*, the
196 isolate analysed here is the PCM2 Trang strain, which is not *L. martiniquensis* (Pothirat et
197 al., 2014). Within the *L. enriettii* complex the most closely related species to the Ghanaian
198 parasites is "*L. siamensis* PCM2 Trang" (98.29% identity, 460/468 nucleotides) followed by
199 *L. enriettii* (90.26% identity) according to the RPL23a tree. Finally we also generated
200 sequences for the RNA polymerase II large subunit gene (RNA PolII; *L. major*
201 chromosome 31), a conserved single copy housekeeping gene that has also been
202 previously used to construct *Leishmania* phylogenies (Croan et al., 1997; Noyes et al.,
203 2002; Yurchenko et al., 2006; Dougall et al., 2011; Pothirat et al., 2014). The sequences
204 from the three isolates were again identical to each other and the resulting ML tree is
205 shown in Figure 3B. This tree is topologically very similar to Figure 3A, the main groups
206 were well supported, and again the Ghanaian isolates clustered within the *L. enriettii*
207 complex. In this tree the most closely related species to the Ghanaian parasites appears
208 different and to be *L. enriettii* (98.42% identity, 1181/1200 nucleotides) followed by "*L.*

209 *siamensis*" (98.08% identity), however, there is relatively low bootstrap support (48%) on
210 the branch including *L. enriettii*. Thus the sequences derived for both the RPL23a
211 intergenic and RNA Pol II sequences were identical between the three isolates, but
212 different from all the *Leishmania* species examined, which includes all the major human
213 pathogens.

214 The phylogenetic analysis showed the Ghanaian parasites to be members of the *L.*
215 *enriettii* complex, a possible new subgenus of *Leishmania* parasites. To date, the evidence
216 for a new subgenus is by inference from phylogenetic analyses such as those presented
217 here and previously (Dougall et al., 2011; Pothirat et al., 2014). Members of the *L. enriettii*
218 complex are clearly not placed within any of the existing subgenera, *L. (Leishmania)*, *L.*
219 *(Viannia)* or *L. (Sauroleishmania)*, and the root of the branch leading to the *L. enriettii*
220 complex is deep and of similar separation to the other subgenera. However, many
221 important details of the biology of these parasites that would be required to make a precise
222 definition of the possible subgenus are still unknown, so until there is progress in that
223 regard this proposition remains to be decided. For example, there is little to no current
224 information on natural reservoir hosts for any species in the *L. enriettii* complex. In both ML
225 trees the location of the Ghanaian isolates within the *L. enriettii* complex was supported by
226 high bootstrap values. The most closely related species are *L. enriettii* and "*L. siamensis*",
227 but the extent of the sequence variation clearly discriminates these species from each
228 other and the Ghanaian parasites. Taking the RNA PolIII gene as an example, the extent of
229 sequence identity between the Ghanaian parasites and "*L. siamensis*" is 98.08%, which is
230 less than between *L. major* and *L. tropica* (98.75%), or *L. braziliensis* and *L. panamensis*
231 (98.92%), which most authorities would regard as valid species. Although more work
232 needs to be done and genetic divergence is not the only consideration, the data currently
233 available supports the notion that the Ghanaian parasites are a distinct species of
234 *Leishmania*. *L. enriettii* is not a human pathogen, having only been isolated from domestic

235 guinea pigs in southern Brazil (Lainson, 1997), whereas "*L. siamensis*" is a human
236 pathogen (Bualert et al., 2012). Of the other two known members of the *L. enriettii*
237 complex, the un-named species from Australia is also not a human pathogen, having only
238 been found in kangaroos and other macropods (Rose et al., 2004; Dougall et al., 2009),
239 whereas *L. martiniquensis* causes human disease manifesting as both CL and visceral
240 leishmaniasis (Desbois et al., 2014; Pothirat et al., 2014; Liautaud et al., 2014). Allied to
241 this, the *L. enriettii* complex consistently appears as the most basal clade within the
242 *Leishmania*, excluding *paraleishmania*, an informal grouping that requires re-classification
243 but not regarded as *Leishmania* species sensu stricto (Cupolillo et al., 2000). Thus what
244 emerges is an early diverging group of parasites that has become geographically widely
245 dispersed, distributed among a wide diversity of hosts, but including some species with the
246 potential to cause human disease. One of the most intriguing aspects of the *L. enriettii*
247 complex is the identity of the vectors responsible for transmission, which have not been
248 established with certainty for any species. However, recent evidence indicates that day-
249 biting midges are responsible for transmitting leishmaniasis to kangaroos in Australia
250 (Dougall et al., 2011). Therefore, it is possible that midges may be involved in transmission
251 of the Ghanaian parasites, despite the presence of candidate sand flies.

252 Cumulatively these data demonstrate that the GH5, GH10 and GH11 isolates are
253 representative of a new human-infective *Leishmania* species in Ghana. Given the previous
254 report of one case of *L. major* infection (Fryauff et al., 2006) we cannot exclude the
255 possibility that other *Leishmania* species may be present in this endemic focus, and
256 identification of further human isolates is required to assess this possibility. However, the
257 evidence for the presence of other species is currently not strong, and typically only one
258 species is found in a particular landscape and ecological niche (Ready, 2013), although
259 multiple species may be geographically sympatric, even if not typically present in exactly
260 the same ecological niche. However, it would not be surprising if this proposed new

261 species is responsible for the majority or even all of the CL cases in the Ho region of
262 Ghana. The local name agbamekanu is also of interest, meaning "gift from somebody who
263 has returned from a journey", and refers to the local belief that the disease has been
264 brought in from neighbouring Togo, travel across the border between the Volta region and
265 Togo being quite frequent. Whether this implied importation of CL is true remains
266 uncertain, but other than young children, when the disease is found in older children and
267 adults it is frequently the case that they are newcomers to the area. Thus the current
268 pattern of infection more likely reflects an exposure of naïve individuals to what has
269 become an established endemic focus in Ghana. Many aspects of this new focus require
270 investigation, including the nature of the vector and presumed animal reservoir hosts, and
271 a proper understanding of epidemiology, but these are important so that appropriate
272 control measures can be considered to help those afflicted by agbamekanu.

273

274 **Acknowledgements**

275 This work received support from Department of Microbiology, Parasitology Unit of the
276 University of Ghana Medical School, The Wellcome Trust grant number 090678/Z/09/Z
277 (GK-N,PAB) and the Leverhulme Trust/Royal Society Africa Award scheme (DB,PAB). The
278 funders had no role in study design, data collection and analysis, decision to publish, or
279 preparation of the manuscript.

280

281 **References**

- 282 Bualert, L., Charungkiattikul, W., Thongsuksai, P., Mungthin, M., Siripattanapipong, S.,
283 Khositnithikul R, Naaglor T, Ravel C, El Baidouri F, Leelayoova S. 2012.
284 Autochthonous disseminated dermal and visceral leishmaniasis in an AIDS patient,
285 southern Thailand, caused by *Leishmania siamensis*. Am. J. Trop. Med. Hyg. 86,
286 821-824.
- 287 Croan, D.G., Morrison, D.A., Ellis, J.T., 1997. Evolution of the genus *Leishmania* revealed
288 by comparison of DNA and RNA polymerase gene sequences. Mol. Biochem.
289 Parasitol. 89, 149-159.
- 290 Cupolillo, E., Medina-Acosta, E., Noyes, H., Momen, H., Grimaldi Jr., G., 2000. A Revised
291 Classification for *Leishmania* and *Endotrypanum*. Parasitol. Today 16, 142-144.
- 292 da Silva, E.S., Gontijo, C.M., Pacheco, R. da S., Brazil, R.P., 2004. Diagnosis of human
293 visceral leishmaniasis by PCR using blood samples spotted on filter paper. Genet.
294 Mol. Res. 3, 251-257.
- 295 Degrave, W., Fernandes, O., Campbell, D., Bozza, M., Lopes, U., 1994. Use of molecular
296 probes and PCR for detection and typing of *Leishmania* - a mini-review. Mem. Inst.
297 Oswaldo Cruz 89, 463-469.
- 298 Desbois, N., Pratlong, F., Quist, D., Dedet, J.P., 2014. *Leishmania (Leishmania)*
299 *martiniquensis* n. sp. (Kinetoplastida: Trypanosomatidae), description of the
300 parasite responsible for cutaneous leishmaniasis in Martinique Island (French West
301 Indies). Parasite 21, 12.
- 302 Dougall, A., Shilton, C., Low Choy, J., Alexander, B., Walton, S., 2009. New reports of
303 Australian cutaneous leishmaniasis in Northern Australian macropods. Epidemiol.
304 Infect. 137, 1516-1520.

305 Dougall, A.M., Alexander, B., Holt, D.C., Harris, T., Sultan, A.H., Bates, P.A., Rose, K.,
 306 Walton, S.F., 2011. Evidence incriminating midges (Diptera: Ceratopogonidae) as
 307 potential vectors of *Leishmania* in Australia. *Int. J. Parasitol.* 41, 571-579.

308 Faulde, M., Schrader, J., Heyl, G., Arnirih, M., Hoerauff, A., 2007. Zoonotic cutaneous
 309 leishmaniasis outbreak in Mazar-e Sharif, northern Afghanistan: An epidemiological
 310 evaluation. *Int. J. Med. Microbiol.* 298, 543-550.

311 Fryauff, D.J., Hanafi, H.A., Klena, J.D., Hoel, D.F., Appawu, M., Rogers, W., Puplampu,
 312 N., Odoom, S., Kweku, M., Koram, K., Wilson, M.D., Raczniak, G., Boakye, D.,
 313 2006. Short report: ITS-1 DNA sequence confirmation of *Leishmania major* as a
 314 cause of cutaneous leishmaniasis from an outbreak focus in the Ho district,
 315 southeastern Ghana. *Am. J. Trop. Med. Hyg.* 75, 502-504.

316 Kweku, M.A., Odoom, S., Puplampu, N., Desewu, K., Kwakye-Nuako, G., Gyan, B.,
 317 Raczniak, G., Kronmann, K.C., Koram, K., Botero, S., Boakye, D., Akuffo, H., 2011.
 318 An outbreak of suspected cutaneous leishmaniasis in Ghana: lessons learnt and
 319 preparation for future outbreaks. *Glob. Health Action* 4, 5527.

320 Lainson, R., 1997. On *Leishmania enriettii* and other enigmatic *Leishmania* species of the
 321 neotropics. *Mem. Inst. Oswaldo Cruz* 92, 377–387.

322 Liautaud, B., Vignier, N., Miossec, C., Plumelle, Y., Kone, M., Delta, D., Ravel, C., Cabié,
 323 A., Desbois, N., 2014. First case of visceral leishmaniasis caused by *Leishmania*
 324 *martiniquensis*. *Am. J. Trop. Med. Hyg.* 14-0205.

325 Mendonca, M.G., de Brito, M.E.F., Rodrigues, E.H.G., Bandeira, V., Jardim, M.L., Abath,
 326 F.G., 2004. Persistence of *Leishmania* parasites in scars after clinical cure of
 327 American cutaneous leishmaniasis: Is there a sterile cure? *J. Infect.* 189, 1018-
 328 1023.

329 Meredith, S.E.O., Zijlstra, E.E., Schoone, G.J., Kroon, C.C.M., van Eys, G.J.J.M.,
 330 Schaeffer, K.U., el-Hassan, A.M., Lawyer, P.G., 1993. Development and application

331 of the polymerase chain reaction for the detection and identification of *Leishmania*
332 parasites in clinical material. Arch. Inst. Pasteur Tunis 70, 419–431.

333 Noyes, H., Pratlong, F., Chance, M., Ellis, J., Lanotte, G., Dedet, J.P., 2002. A previously
334 unclassified trypanosomatid responsible for human cutaneous lesions in Martinique
335 (French West Indies) is the most divergent member of the genus *Leishmania* ss.
336 Parasitol. 124, 17-24.

337 Nzelu, C.O., Kato, H., Puplampu, N., Desewu, K., Odoom, S., Wilson, M.D., Sakurai, T.,
338 Katakura, K., Boakye, D.A., 2014. First detection of *Leishmania tropica* DNA and
339 *Trypanosoma* species in *Sergentomyia* sand flies (Diptera: Psychodidae) from an
340 outbreak area of cutaneous leishmaniasis in Ghana. PLoS Neg. Trop. Dis. 8,
341 e2630.

342 Okwori, E.N.A., Nock, I.H., Galadima, M., Ibrahim, S., 2001. Prevalence of cutaneous
343 leishmaniasis in parts of Kaduna State, Nigeria. J. Protozool. Res. 11, 32-46.

344 Pothirat, T., Tantiworawit, A., Chaiwarith, R., Jariyapan, N., Wannasan. A., Siriyasatien,
345 P., Supparatpinyo, K., **Bates**, M.D., Kwakye-Nuako, G., **Bates**, P.A., 2014. First
346 isolation of *Leishmania* from northern Thailand: case report, identification as
347 *Leishmania martiniquensis* and phylogenetic position within the *Leishmania enriettii*
348 complex. PLoS Neg. Trop. Dis. 8, e3339.

349 Ready, P.D., 2013. Biology of phlebotomine sand flies as vectors of disease agents. Annu.
350 Rev. Entomol. 58, 227-250.

351 Reithinger, R., Dujardin, J.C., Louzir, H., Pirmez, C., Alexander, B., Brooker, S., 2007.
352 Cutaneous leishmaniasis. Lancet Infect. Dis. 7, 581-596.

353 Rose, K., Curtis, J., Baldwin, T., Mathis, A., Kumar, B., Sakthianandeswaren, A., Spurck,
354 T., Low Choy, J., Handman, E., 2004. Cutaneous leishmaniasis in red kangaroos:
355 isolation and characterisation of the causative organisms. Int J Parasitol. 34, 655–
356 664.

357 van Eys, G.J.J.M., Schoone, G.J., Kroon, N.C.M., Ebeling, S.B., 1992. Sequence analysis
358 of small subunit ribosomal RNA genes and its use for detection and identification of
359 *Leishmania* parasites. Mol. Biochem. Parasitol. 51, 133-142.

360 Villinski, J.T., Klena, J.D., Abbassy, M., Hoel, D.F., Puplampu, N., Mechta, S., Boakye, D.,
361 Raczniak, G., 2007. Evidence for a new species of *Leishmania* associated with a
362 focal disease outbreak in Ghana. Diagn. Microbiol. Infect. Dis. 60, 323-327.

363 Yurchenko, V.Y., Lukes, J., Jirku, M., Zeledón, R., Maslov, D.A., 2006. *Leptomonas*
364 *costaricensis* sp. n. (Kinetoplastea: Trypanosomatidae), a member of the novel
365 phylogenetic group of insect trypanosomatids closely related to the genus
366 *Leishmania*. Parasitol. 133, 537-546.

367

368 **Figure Legends**

369 **Figure 1. PCR diagnosis and RFLP.** A. An example of diagnosis using kDNA minicircle
370 primers. Lesions were cleaned with 70 % alcohol and scrapings stored on FTA cards
371 (Whatman BioScience). Three 2 mm discs from each FTA sample were processed for
372 PCR and the products examined by agarose gel electrophoresis. Numbers 59-65 above
373 lanes refer to participant sample numbers; M1 is a 100 base pair ladder marker; N is a
374 negative control; 357, 546, and FV1 are positive controls for *L. tropica*, *L. aethiopica* and *L.*
375 *major*, respectively. B. An example of diagnosis using 18S rRNA primers. Numbers 11-22
376 refer to participant sample numbers; M1 is a 100 base pair ladder marker; N is a negative
377 control; 357, 546, JPC, LV9 and FV1 are positive controls for *L. tropica*, *L. aethiopica*, *L.*
378 *infantum*, *L. donovani* and *L. major*, respectively. C. Analysis of isolates by PCR-RFLP.
379 DNA was purified from cultures of isolates GH5, GH10, GH11 and positive controls,
380 amplified using primers AM1/AM2, and products digested with restriction enzyme *MspI*. M
381 contains size markers; LV546, LV357 and FV1 are controls for *L. aethiopica*, *L. tropica* and
382 *L. major*, respectively; N is a negative control. Further technical details are given in
383 Supplementary Data S1, and further results in Supplementary Figure S1.

384

385 **Figure 2. Appearance of lesions from confirmed cases of cutaneous leishmaniasis.**

386 Examples of typical active lesions are shown on several participants: A, on the arm; B, C,
387 D, on the head; and E, on the back. In F is an example of the scar developing in a healing
388 lesion.

389

390 **Figure 3. Phylogenetic analysis of Ghanaian *Leishmania*.** A. Maximum likelihood tree
391 based on RPL23a intergenic sequences, with 22 species of *Leishmania* and
392 *Endotrypanum monterogeii*, and using *Crithidia fasciculata* as an outgroup, based on
393 alignment of 405-547 homologous nucleotide sequences. AM-2004 is an un-named

394 species of *Leishmania* from Australia. The accession numbers for the sequences used are
395 given in Table S1 and the various subgenera and other groups are as indicated. Bootstrap
396 values from 1000 replicates are given at the nodes. B. Maximum likelihood tree based on
397 RNA Polymerase II large subunit gene sequences, with 22 species of *Leishmania* and
398 *Endotrypanum monterogeii*, using *Crithidia fasciculata* as an outgroup, based on alignment
399 of 1191-1200 homologous nucleotide sequences. Accession numbers are given in Table
400 S1. Further technical details are given in Supplementary Data S1.

401

402

403 **Supplementary Information Legends**

404

405 **Figure S1. Further examples of PCR diagnosis.** A. Four panels showing PCR results
406 from diagnosis using kDNA minicircle primers. Numbers above lanes refer to participant
407 sample numbers; M1 is a 100 base pair ladder marker; N is a negative control; 357, 546,
408 and FV1 are positive controls for *L. tropica*, *L. aethiopica* and *L. major*, respectively. B.
409 Two panels showing PCR results from diagnosis using 18S rRNA primers. Numbers
410 above lanes refer to participant sample numbers; M1 is a 100 base pair ladder marker; N
411 is a negative control; 357, 546, JPC, LV9 and FV1 are positive controls for *L. tropica*, *L.*
412 *aethiopica*, *L. infantum*, *L. donovani* and *L. major*, respectively.

413

414 **Figure S2. Multiple sequence alignment of ITS1 sequences.** The sequences derived
415 from three independent CL cultures (GH5, GH10 and GH11; accession numbers
416 KP006688-KP006690) are aligned with the sequence from Villinski et al. [17] (TAVE;
417 EF524071), previously attributed to a new species of *Leishmania*. Identical residues are
418 indicated by asterisks and the two variable residues are shaded.

419

420 **Figure S3. Participant recruitment.** Villages were visited through the year, the number of
421 participants recruited into the study during different months is shown.

422

Table S1. Accession numbers of sequences used for phylogenetic analysis.

Species	WHO Code	Accession number for RPL23a IGS	WHO Code	Accession number for RNAPolIII
GH5	MHOM/GH/2012/GH5;LV757	KP006691 ^a	MHOM/GH/2012/GH5;LV757	KP054394 ^a
GH10	MHOM/GH/2012/GH10;LV758	KP006692 ^a	MHOM/GH/2012/GH10;LV758	KP054395 ^a
GH11	MHOM/GH/2012/GH11;LV759	KP006693 ^a	MHOM/GH/2012/GH11;LV759	KP054396 ^a
<i>L. adleri</i>	RLIZ/KE/XXXX/LV30	KP025941 ^a	RLIZ/KE/XXXX/LV30	AF009153
<i>L. amazonensis</i>	MHOM/BR/1997/M2269	FR693777	MHOM/BR/1973/LV78	AF009154
<i>Leishmania</i> new species from Australia	MMAC/AU/2004/AM-2004;Roo1	FR693774	MMAC/AU/2004/AM-2004;Roo1	HM775497
<i>L. braziliensis</i>	MHOM/BR/83/LTB300	FR693776	MHOM/VE/XXXX/LBV	AF009155
<i>L. colombiensis</i>	IHAR/CO/1996/CL500;LEM2334	KP025942 ^a	IHAR/CO/1996/CL500;LEM2334	KM820662
<i>L. donovani</i>	MHOM/ET/67/HU3;L82;LV9	FR693771	MHOM/IN/1980/DD8	AF009157
<i>L. enriettii</i>	MCAV/BR/1945/LV90	FR693773	MCAV/BR/1945/LV90	AF151727
<i>L. equatorensis</i>	MCHO/EC/1982/Lsp1;L888	KP025943 ^a	MCHO/EC/1982/Lsp1;L888	DQ383655
<i>L. gerbilli</i>	MRHO/CN/1960/Gerbilli;LON-25	FR693785	MRHO/CN/1960/Gerbilli;LON-25	AJ304947
<i>L. guyanensis</i>	MHOM/BR/75/M4147	FR693784	MHOM/SR/1987/TRUUSI	AJ304949
<i>L. gymnodactyli</i>	RGEC/SU/XXXX/LV247	FR693781	RGEC/SU/XXXX/LV247	AF009159
<i>L. hertigi</i>	MCOE/PA/1965/C-8;LV42	FR693775	MCOE/PA/1965/C-8;LV42	AF009161
<i>L. hoogstraali</i>	RLIZ/SD/XXXX/LV31	FR693782	RLIZ/SD/XXXX/LV31	AF009162
<i>L. infantum</i>	MCAN/ES/1998/LEM-935;JPCM5	FR796438	MCAN/ES/1998/LEM-935;JPCM5	XM_001467548
<i>L. major</i>	MHOM/IL/1980/Friedlin;FV1	FR796402	MHOM/IL/1980/Friedlin;FV1	XM_001685196
<i>L. martiniquensis</i>	MHOM/MQ/1992/MAR1; LEM2494	KP025945 ^a	MHOM/MQ/1992/MAR1; LEM2494	KM820663
<i>L. mexicana</i>	MNYC/BZ/1962/M379	FR693769	MNYC/BZ/1962/M379	AF009164
<i>L. panamensis</i>	MHOM/PA/XX/53A;LV145	FR693783	MHOM/PA/XXXX/CIDEP004	AF009165
" <i>L. siamensis</i> "	MHOM/TH/2010/PCM2;Trang	KP025944 ^a	MHOM/TH/2010/PCM2;Trang	KM820664
<i>L. tarentolae</i>	RTAR/SE/67/G10/LV108	FR693780	RTAR/DZ/1939/LV414	AF009166
<i>L. tropica</i>	MHOM/IR/60/LV357	FR693778	MHOM/SU/1958/Strain-OD	AF009167
<i>L. turanica</i>	MRHO/SU/83/MARZ-051	FR693786	IRAI/TR/1991/M87	AJ304946
<i>Endotrypanum monterogeii</i>	MCHO/CR/1962/LV88;A9	FR693788	MCHO/CR/1962/LV88;A9	AF009158
<i>Crithidia fasciculata</i>	Cf-C1	CfaC1_23 571,910-572,533 ^b	Cf-C1	Cfa-C1_34 1,685,828-1,687,024 (minus strand) ^b

^aThose that have been generated as part of this study. ^bTriTrypDB identifier (<http://tritrypdb.org/tritrypdb/>), Stephen M. Beverley and The Genome Institute, Washington University School of Medicine.

Table S2. Age group and gender distribution in confirmed CL cases. Diagnosis was confirmed by PCR from dermal scrapings (38 participants) or by culture and DNA sequencing (3 participants).

Age Group	Sex		Total
	Male	Female	
0-10	14	10	24
11-20	1	1	2
21-30	0	7	7
31-40	0	2	2
41-50	1	0	1
≥51	2	3	5
Total	18	23	41

Table S3. Numbers of lesions on different regions of the body in confirmed CL cases. The majority of cases presented with single lesions (33/41) but 8 individuals had multiple lesions.

Body Region	Number of Lesions
Head	27
Neck	1
Arm	11
Trunk	6
Leg	6
Total	51

Figure 1

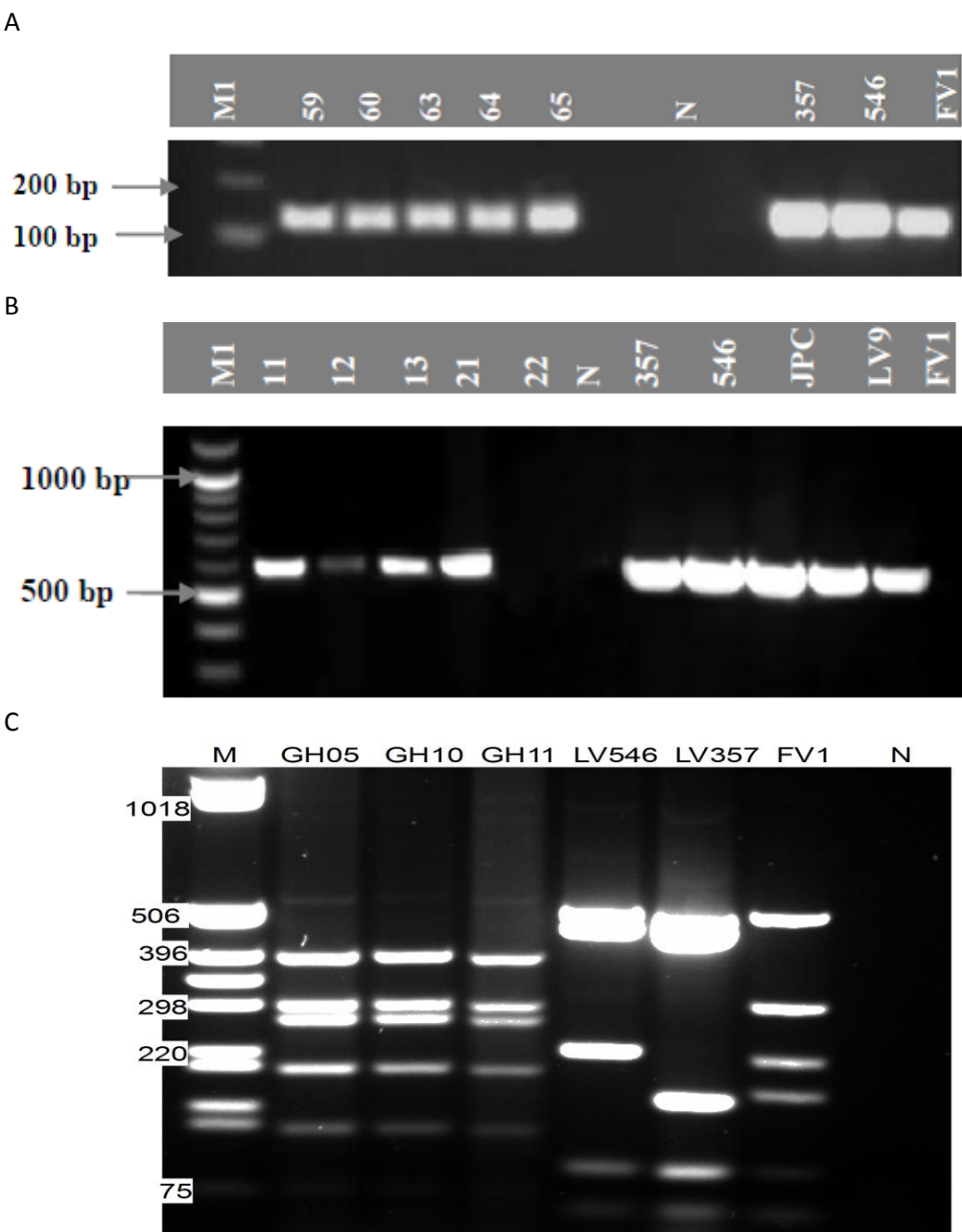


Figure 1

Figure 2



Figure 2

Figure 3

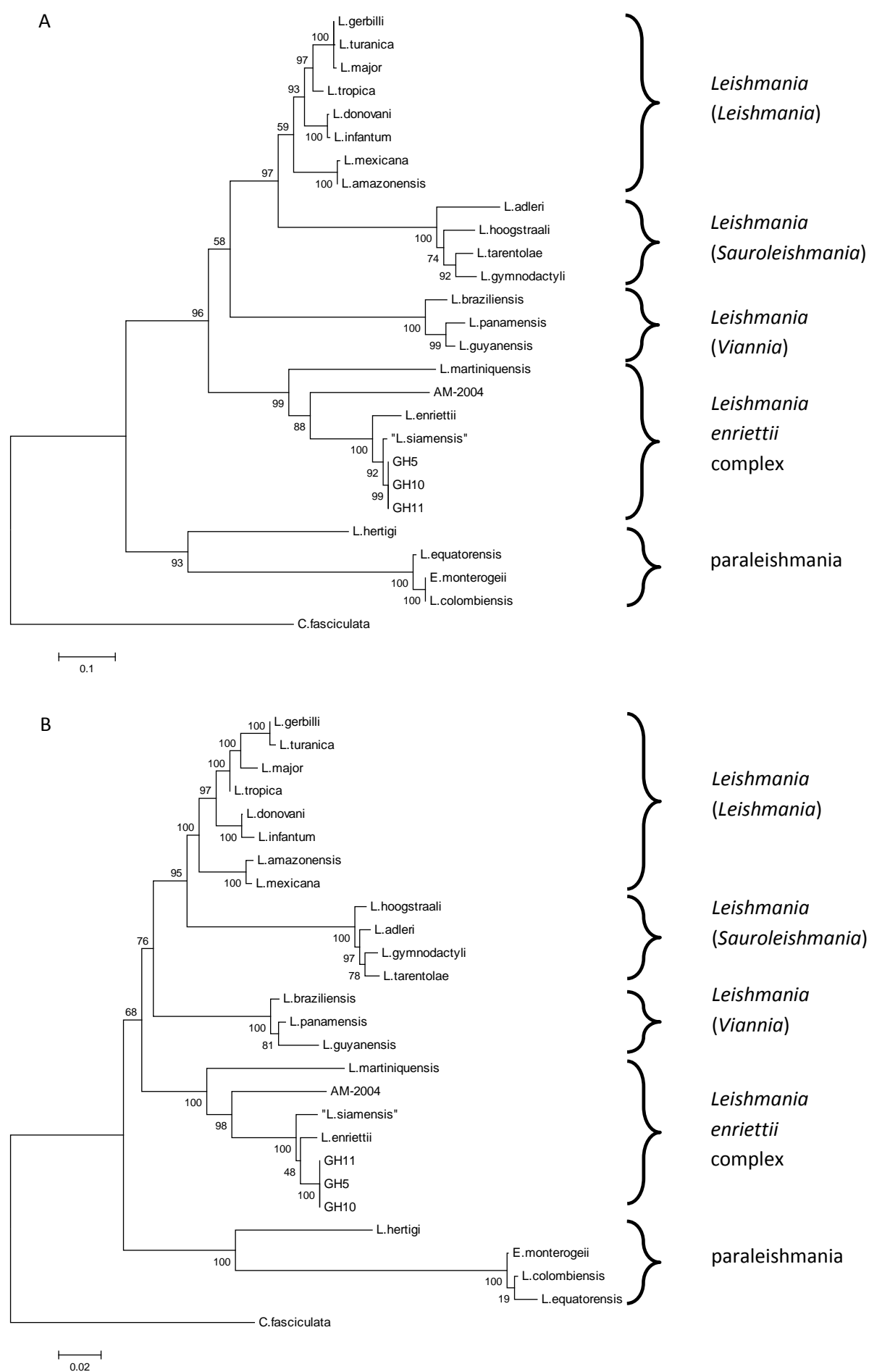
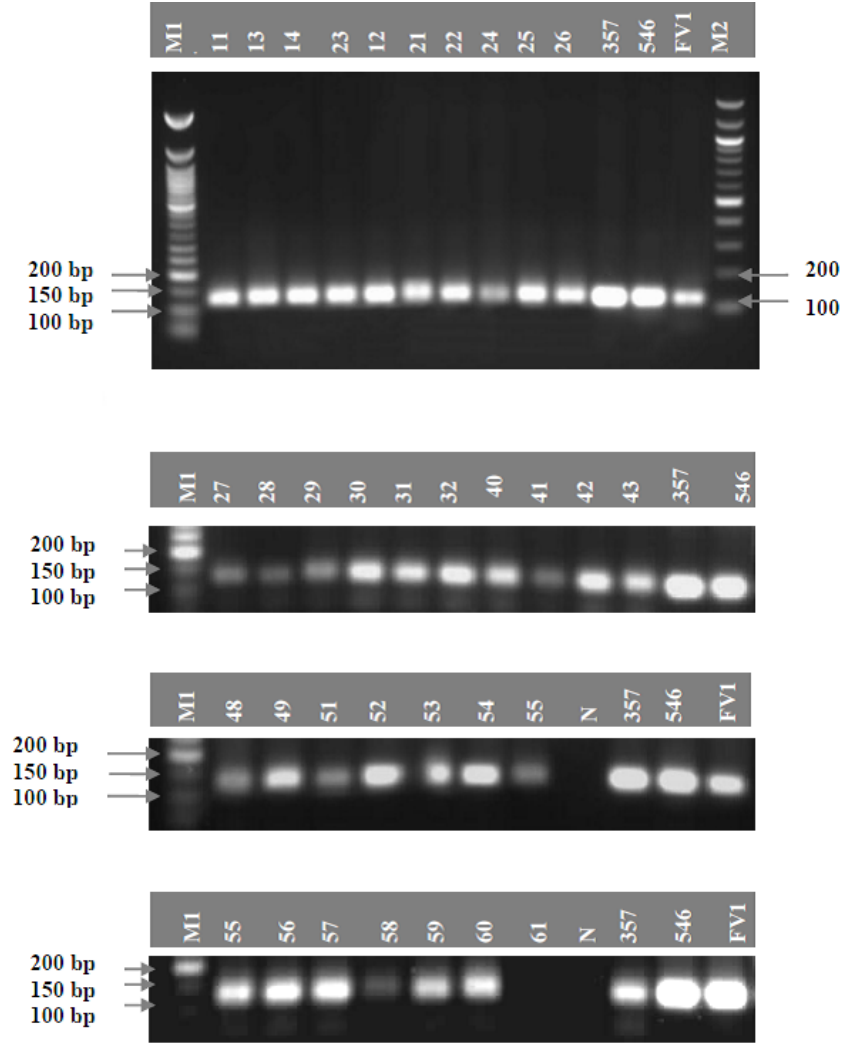


Figure 3

A



B

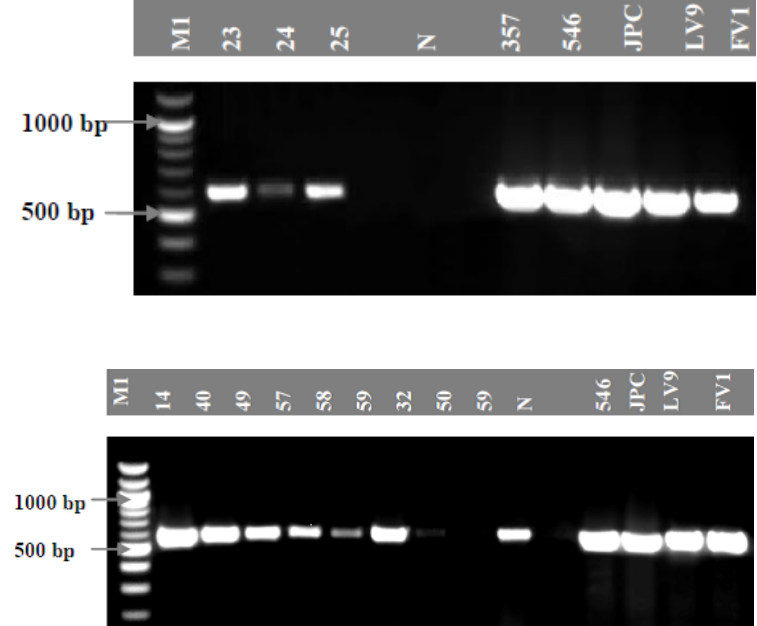


Figure S1

TAVE	ATTACACCAAAAAAAAAACATACAATCAAAACACGGGGAGGTGTATCTCTCTTTTTGTCAG	60
GH5	ATTACACCAAAAAAAAAACATACAATCAAAACACGGGGAGGTGTATCTCTCTTTTTGTCAG	60
GH10	ATTACACCAAAAAAAAAACATACAATCAAAACACGGGGAGGTGTATCTCTCTTTTTGTCAG	60
GH11	ATTACACCAAAAAAAAAACATACAATCAAAACACGGGGAGGTGTATCTCTCTTTTTGTCAG	60

TAVE	ATAACGCCTTTCCACATACACACACAACAATATATATGTATATATGTATTGTTATACTC	120
GH5	ATAACGCCTTTCCACATACACACACAACAATATATATGTATATATGTATTGTTATACTC	120
GH10	ATAACGCCTTTCCACATACACACACAACAATATATATGTATATATGTATTGTTATACTC	120
GH11	ATAACGCCTTTCCACATACACACACAACAATATATATGTATATATGTATTGTTATACTC	120

TAVE	AATTATACAGTAAACAGAAAAACAAAGGCCGGTCGACATATACACACCGCGCGTATATAT	180
GH5	AATTATACAGTAAACAGAAAAACAAAGGCCGGTCGACATATACACACCGCGCGTATATAT	180
GH10	AATTATACAGTAAACAGAAAAACAAAGGCCGGTCGACATATACACACCGCGCGTATATAT	180
GH11	AATTATACAGTAAACAGAAAAACAAAGGCCGGTCGACATATACACACCGCGCGTATATAT	180

TAVE	ATATATACAACGAAAATGTCCCTTCTTACGGGGGCTTTTCTGGCGGTGTGCTGTGGATAA	240
GH5	ATATATACAACGAAAATGTCCCTTCTTACGGGGGCTTTTCTGGCGGTGTGTTGTGGATAA	240
GH10	ATATATACAACGAAAATGTCCCTTCTTACGGGGGCTTTTCTGGCGGTGTGTTGTGGATAA	240
GH11	ATATATACAACGAAAATGTCCCTTCTTACGGGGGCTTTTCTGGCGGTGTGTTGTGGATAA	240

TAVE	CGGCTCACAT	250
GH5	CGGCTCACAT	250
GH10	CGGCTCACAT	250
GH11	CGGCTCACAT	250

Figure S2

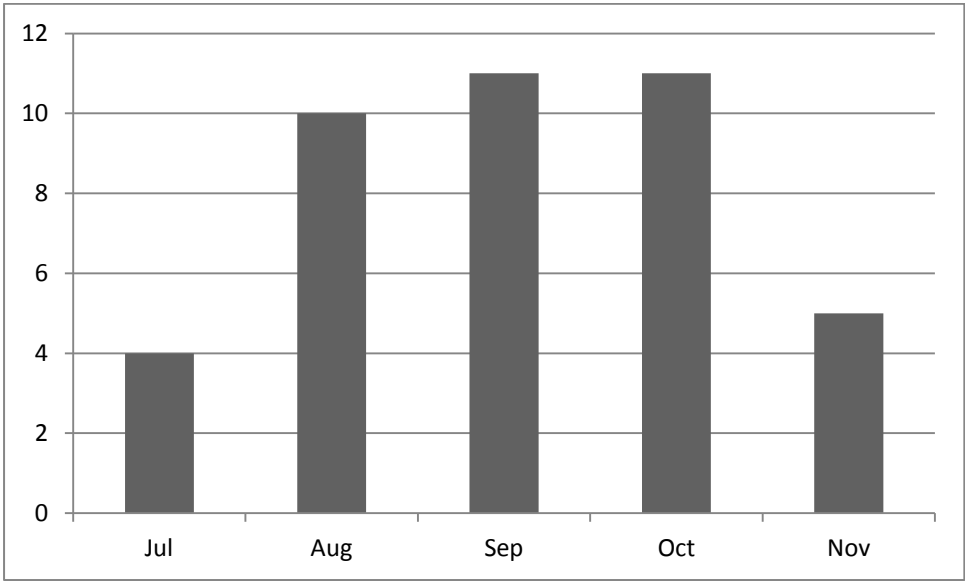


Figure S3

Supplementary Data S1*Sample collection*

Skin scrapings were collected from the site of an active lesion using a single-use sterile scalpel. The surfaces and about 30 mm around the diameter of the lesions were cleaned with 70 % alcohol. The scrapings obtained from the borders and the centre of the ulcerated lesions were transferred into 150 µl of phosphate buffered saline (PBS) contained in 1.5 ml microfuge tubes. The PBS and their contents were then spotted onto FTA cards (Whatman BioScience WB12 0205) and air-dried. Samples were stored in closed plastic bags and kept under dry conditions at room temperature to preserve DNA for later PCR analysis. Alternatively, lesions were sterilized with 70 % alcohol, anaesthetized with 1 % lidocaine and sampled by needle aspiration. Aspirates were placed into bijoux tubes containing 2 ml of Hank's balanced salt solution (Life Technologies 14170-112) and 25 µg/ml gentamicin sulphate, and stored on ice until processing in the laboratory. Any bleeding was controlled and a sterile dressing applied. All patients received treatment against secondary infections with topical antibiotics and antifungal creams.

PCR using FTA discs

Samples were prepared according to the manufacturer's instructions. At least three 2 mm discs from different sites on the same sample spot of the FTA card were excised using a carbon steel surgical blade and placed in PCR amplification tube. A single blade was used for each sample to prevent cross contamination between samples. The excised discs were then suspended in 200 µl of FTA purification reagent (Whatman Bioscience WB12 0204) in the PCR tube and incubated for 5

minutes at room temperature. The FTA Purification Reagent was then removed and discarded. This washing step was repeated for a total of 3 washes with FTA purification reagent. After the third wash, the discs were re-suspended in 200 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and incubated for 5 minutes at room temperature, after which the TE buffer was removed and discarded. The washing procedure with TE buffer was repeated three times. Finally, the discs were allowed to dry at room temperature for 1 hour. PCR reagents were then added directly to the discs in the tube. Primers Mincr2 (5' GGG GAG GGG CGT TCT GCG AA 3') and Mincr3 (5' CGC CCC CTA TTT TAC ACA ACC CC 3') were used in an optimized PCR reaction mixture with conditions as follows: 25 µl volumes were prepared using 2 x Reddy Mix™ PCR Master Mix (ABgene AB-0575/DC/LD) (1.5 mM MgCl₂, 1.25 units *Taq* DNA polymerase, 75 mM Tris-HCl (pH 8.8 at 25 °C), 20 mM (NH₄)₂ SO₄, 0.01 % (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP, dTTP, and Precipitant and red dye for electrophoresis), 100 pmol of each primer and 2 mm incised discs of the FTA card. Initial denaturation was at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 1 minute 30 seconds, and a final extension at 72 °C for 3 minutes. Primers R221 (5' GGT TCC TTT CCT GAT TTA CG 3') and R332 (5' GGC CGG TAA AGG CCG AAT AG 3') were used in an optimized PCR reaction mixture on FTA discs with conditions as above, except that a 1.1 x master mix with 2.5 mM MgCl₂ was used (Abgene AB-0619/LD) and a 63 °C annealing temperature.

PCR on cultured parasites

DNA extraction from parasites was performed using a QIAamp DNA Mini Kit (Qiagen), following the manufacturer's instructions. Amplification of the ITS1

sequence was performed using primers L5.8S (TGA TAC CAC TTA TCG CAC TT) and LITSR (CTG GAT CAT TTT CCG ATG). Amplification of the RPS7 intergenic sequence was performed using primer pairs AM1 (CGC GTG TCG TTC GGC TTT ATG TG) and AM2 (CTT ACG GAG CTT GCT GAG GTG AGG), followed by digestion with restriction enzyme *MspI*. Amplification of the RPL23a intergenic sequence was performed using primers BN1 (GAA GGT CAA CAC CCT GAT CC) and BN2 (CTT CTT GGC GGT CTT CTG AG). Amplification of RNA Polymerase II was performed with several primer pairs: RPOF1 (GAC ACA GCC GTC AAG AC) and RPOR1 (GCA GCC GCA CAA TGC GCT); PolIIIN5 (GCA CTT CAT GTT GGA CGA CT) and PolIIIN6 (GTA CTT GGT GCG GAT CTC CT); PolIIIN7 (AGG AGT ACA GGC TGA ACG AC) and PolIIIN8 (TGT CGT CCA CTT GCC GGA). Amplification was performed with proof-reading DNA polymerase (Qiagen HotStar HiFidelity Polymerase).

Agarose gel electrophoresis

PCR products were loaded onto 8 cm 1.5 % - 2 % agarose gels containing 5 ng/μl ethidium bromide and Tris-acetate (TAE) buffer and electrophoresed for 45 minutes to 1 hour at 75 – 100 V. DNA bands were examined under ultraviolet trans-illumination and their size determined by comparison with markers. Alongside test samples, positive controls were run including products generated from *L. donovani* (MHOM/ET/67/HU3; LV9); *L. infantum* (MCAN/ES/98/LEM-935; JPC; M5); *L. tropica* (MHOM/IR/60/LV357); *L. major* (MHOM/IL/80/Friedlin; FV1); and *L. aethiopica* (MHOM/ET/72/LRC-L147/546).

DNA sequencing and phylogenetic analysis

Products were directly sequenced using commercial services. Results were checked for quality using Chromas Lite 2.1.1 (<http://technelysium.com.au/>). Initial alignments and analyses were performed using Clustal W2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). For phylogenetic analysis, alignment and tree building programmes in MEGA version 6 were used (<http://www.megasoftware.net/>). Testing of alternative models was performed, for RPL23a sequences the Hasegawa-Kishino-Yano model and for RNA polymerase the Tamura-Nei model were the best fitting models of sequence evolution, respectively, and these were used for tree construction using the maximum likelihood method. Bootstrapping was performed on all trees with 1000 replicates.